Characterizing effects of sumoylation on Thymine-DNA Glycosylase base excision repair activity *in vivo*

by

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Abstract

Thymine-DNA glycosylase (TDG) is an enzyme that recognizes and repairs G/T and G/U mismatches in the base excision repair (BER) pathway. Its BER function is important in maintaining genome integrity and in regulating DNA gene expression through DNA demethylation of 5-methylcystosine (5mC). It has been proposed that dynamic sumoylation-desumoylation is required for efficient enzymatic turnover of TDG *in vitro*. TDG sumoylation alleviates product inhibition at the abasic site after enzyme processing and allows dissociation of TDG from DNA. Based on this model, subsequent desumoylation of TDG is required for complete enzymatic turnover. The effect of sumoylation on TDG activity in vivo, however, has not been studied. Here we have devised an in vivo assay to study the role of sumoylation in regulating TDG activity. Ten-eleven translocation (TET) enzyme iteratively oxidizes 5-mC of genomic DNA to a final product of 5-carboxylcytosine (5caC). Because TDG is the only enzyme capable of recognizing and repairing 5caC, we co-expressed TDG sumoylation mutant constructs with TET in HEK293T cells and measured 5caC levels of genomic DNA to monitor TDG activity. Our results showed that sumoylation does not affect TDG BER activity *in vivo*. We also characterized the specific-SUMO isopeptidases involved in TDG desumoylation. Co-expressing SENP1 and 2 constructs with wild-type TDG demonstrated that SENP1 preferentially desumoylates TDG. Additionally, only the catalytic domain of SENP1 is required for efficient TDG desumoylation. Our findings provide important insight into the molecular mechanisms that regulate key biological processes involving TDG activity such as gene regulation, development, and maintenance in genomic integrity.

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Table of Contents

Introduction

Regulation and coordination of DNA repair mechanisms is an essential aspect of maintaining genome integrity and ensuring proper cell function. Sumoylation is a post-translational modification that regulates a number of cellular processes, such as governing mitotic progression by influencing chromosomal segregation (Cubenas-Potts, Goeres, & Matunis, 2013) as well as facilitating protein degradation through RNF4-dependent proteasome targeting (Sriramachandran & Dohmen, 2014). Sumoylation regulates specific aspects of DNA replication, repair, and transcription (Ulrich, 2014). From promoting DNA break repair through Ku70 (Hang et al., 2014), to determining robustness of DNA repair response by localization of PTEN (Bassi et al., 2013), sumoylation has a clear role in regulating many different DNA repair pathways. However, the molecular factors determining sumoylation in response to damage and desumoylation following repair of these DNA repair proteins are not fully understood.

Sumoylation is a post-translational modification involving the attachment of the 15 kDa protein, small ubiquitin-like modifier (SUMO), to a target protein (Geiss-Friedlander & Melchior, 2007). SUMO imparts protein regulation either by covalent attachment of SUMO to the target or by mediating protein-protein interactions via a SUMO-interacting motif (SIM) contained on a protein that interacts non-covalently with a SUMO conjugated to another protein. There are four paralogs of SUMO protein: SUMO1, SUMO2, SUMO3, and SUMO4. SUMO2 and SUMO3 have ~95% identity and are currently indiscernible at the protein level and are thus termed SUMO2/3. SUMO2/3 has ~45% identity with SUMO1 and are functionally distinct as SUMO2/3 has preference for forming chains while SUMO1 tends to prefer monosumoylation. SUMO4 does not appear to be expressed on the protein level in normal cells and is therefore generally overlooked. Like ubiquitination, sumoylation is mediated by an E1 (Aos1/Uba2)/E2

(Ubc9)/E3 (several) cascade that results in the formation of a covalent bond between the C-terminal glycine of the SUMO protein and the ε-amino of the target protein lysine. However unlike ubiquitination, only one E2 and a handful of E3's have been identified in SUMO conjugation. This suggests a greater role for the deconjugating enzymes in sumoylation (Yeh, 2009). Sentrin-specific proteases (SENP) are the major family of desumoylating enzymes and the best studied (Mukhopadhyay & Dasso, 2007). Efficient protein sumoylation requires the dynamic process of conjugation and deconjugation. Interestingly, the majority of proteins that are sumoylated are only modified to a very small fraction of the total protein pool suggesting great importance of the dynamic nature of sumoylation. Determining the SENP isopeptidases responsible for deconjugating modified proteins, as well as determining the E3s responsible for conjugation, is a major step in better understanding the dynamics of sumoylation for a given protein and ultimately the regulatory role of the SUMO modification. This is particularly important as dysregulation in SUMO signaling is increasingly appreciated to affect health and contribute to human disease (Flotho & Melchior, 2013)

Thymine-DNA glycosylase (TDG) is a monofunctional glycosylase DNA repair protein that is sumoylated. Specifically, TDG participates in base excision repair (BER), recognizing G/U and G/T mismatches arising from spontaneous cytosine deamination and nucleotide misincorporation, respectively (Barrett et al., 1998; Lari, Al-Khodairy, & Paterson, 2002). TDG-specific BER is of particular interest because of the characteristic product inhibition observed by the glycosylase at the abasic site (Hardeland et al., 2001; Waters, Gallinari, Jiricny, & Swann, 1999). Generally, BER proceeds when a glycosylase recognizes a specific DNA lesion then excises the lesion producing an abasic site. Following excision, apurinic/apyrimidinic endonuclease 1 (APE1) displaces the glycosylase and creates a single strand break at the abasic site. Then, either by short patch or long patch sub-pathways, correct nucleotides are incorporated and the DNA is

ligated and repair is completed (Figure 1). However, more robust binding of TDG to the abasic site than to the DNA lesion results in product inhibition and the inability to progress through BER. Sumoylation has been proposed as the mechanism of alleviating this product inhibition (Hardeland, Steinacher, Jiricny, & Schar, 2002).

TDG contains a N-terminal SIM at residues 133-137, a C-terminal SIM at residues 308-311, and a SUMO-conjugation site at residue K330 (Figure 2A). Interestingly, when TDG is conjugated by either SUMO1 or SUMO3 at K330, the SUMO protein interacts non-covalently with the adjacent C-terminal SIM at E310 (Baba et al., 2005, 2006). This intramolecular SUMO-interaction leads to a conformational change in the N-terminus of modified TDG resulting in the formation of a protruded α-helix that is unstructured in unmodified TDG (Smet-Nocca, Wieruszeski, Leger, Eilebrecht, & Benecke, 2011). The protruded α-helix is proposed to affect BER activity by disrupting TDG binding to DNA, promoting TDG dissociation and thus alleviating product inhibition (Figure 2B) (Steinacher & Schar, 2005). Based on these findings, sumoylation is proposed to regulate TDG activity by decreasing affinity for abasic DNA through a conformational change in the protein. However, this work has been questioned recently by evidence that TDG is not preferentially sumoylated when bound to DNA and that APE1 may be sufficient to disrupt product inhibition (Coey et al., 2014; Fitzgerald & Drohat, 2008). This suggests that sumoylation may not be necessary for efficient catalytic turnover of TDG at the abasic site. Additionally, the majority of the evidence for this paradigm was performed *in vitro* and has yet to be interrogated *in vivo*.

In this study, we investigated the role of sumoylation in regulating TDG activity *in vivo*. We developed an *in vivo* BER activity assay using a cell culture system and determined the effects of different sumoylation TDG mutations on TDG BER activity. We took advantage of the recent discoveries that ten-eleven translocation family enzymes (TET) convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), 5-

formylcytosine (5fC), and 5-carboxylcytosine (5caC) (Ito et al., 2010; Ito et al., 2011) in an iterative oxidation pathway and that 5fC and 5caC are specific substrates for TDG BER activity (He et al., 2011; Kohli & Zhang, 2013; Maiti & Drohat, 2011; Shen et al., 2013). While the emerging importance of TET and TDG working jointly in an active DNA demethylation pathway is appreciated, we focused only on the unique and specific nature of TET enzymes producing substrates that only TDG can repair in order to assay TDG activity (Figure 3). Our findings suggest that neither SUMO conjugation nor SUMO interactions regulated TDG BER activity *in vivo*.

Additionally, we sought to determine the SENP responsible for desumoylating TDG in order to further test the model of sumoylation regulating TDG BER activity. If the model holds that sumoylation is required in response to damage to alleviate TDG product inhibition at the abasic site, then desumoylation following repair is necessary to prime TDG for another cycle of DNA damage response. Using a library of SENP constructs and mutants in cell culture, we found that SENP1 specifically desumoylates SUMO-TDG and that the catalytic domain is sufficient for this desumoylation. Taken together, we demonstrate that TDG sumoylation is tightly regulated by SUMO machinery but that sumoylation regulates an aspect of TDG biology other than BER activity.

Materials and Methods

Cell culture and transient transfections

U2OS and HEK293T cells were cultured in Dulbecco's modified Eagle's medium-high glucose (DMEM-1X, Life Technologies, cat. 11965-092) containing 4.5 g/L D-glucose and L-glutamine and supplemented with 10% fetal bovine serum, 1% HEPES, phenol red, and antibiotics and maintained at 37°C in 5% CO_2 incubator. For transient transfections, cells were plated in 6-well plates with supplemented DMEM-1X culture media absent antibiotics. Cells were transfected with plasmids at confluency of 40% using X-tremegene HP transfection reagent (Roche Diagnostics) for transient overexpressions or Lipofectamine RNAiMAX (Life technologies) for transient knockdowns per manufacturers' instructions. DNA plasmids were up brought to 1 µg total DNA. RNAi oligos were used at final concentrations of 20 nM.

Plasmid and oligo constructs

TDGWT and TET1cd expression constructs were a gift from Alex Drohat of University of Maryland School of Medicine. TDGWT was cloned into pcDNA3.0 expression vector with an N-terminal FLAG tag. TDGK330R, TDGE310Q, and TDGN140A constructs were generated by site-directed mutagenesis. TET1cd resides on a pLEXm expression vector and contains an N-terminal His6 tag. SENP1, SENP1 catalytic mutant, SENP2, SENP1 and 2 chimeras, and SENP1 deletion constructs were generated as previous described (Cubenas-Potts et al., 2013). All SENP constructs were expressed in pEGFP-C1 vectors. SUMO1 expression construct resides on pcDNA3.0 vector and contains an N-terminal Myc tag. siRNA oligos used were as follows: 5`-UCCUUUACACCUGUCUCGAUGUCUU-3` for SENP1 and 5`-

GAAAGAGAGAAGUACCGAAtt-3` for SENP2. The commercially available NC1 oligo (Integrated DNA Technologies) was used as a mock siRNA.

Whole cell lysate preparation

For whole cell lysate preparations, cells were washed once with 1X phosphate-buffered saline (PBS) then lysed with 100 µL (for 6-well plate) or 600 µL (for 10 cm dish) modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCL, 1% NP40, 0.25% sodium deoxycholate, 1 mM EDTA, 0.1% SDS) supplemented with inhibitors 1 mM phenylmethylsulfonyl (PMSF), 5 mM N-ethylmaleimide (NEM), and 1X SigmaFAST protease inhibitor cocktail (Sigma-Aldrich, cat. S8830). Some experiments used an alternative lysis of TritonX lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% TritonX-100) with the same supplements. Additionally, some experiments did not include the supplementation of NEM in either lysis condition. After incubation for 30 minutes at 4°C, lysates were sonicated (3 output level, 5 seconds), centrifuged (14,000 rpm, 4°C, 30 minutes), and stored in -80°C. Protein concentrations of all whole cell lysates were measured by bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific).

Western blot

Prepared whole cell lysates were brought up in suspension with 5X SDS loading buffer (300 mM Tris-HCl pH 6.8, 10% SDS, 50% glycerol, 500 mM 2-mercaptoethanol, and 0.05% bromophenol blue). Suspension was then incubated for 10 minutes at 96°C. For Western blotting, proteins were separated in 10% or 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membrane (0.45 µm, Bio-Rad, cat. 1620115). All membranes were blocked with 5% dry milk in 1X Tris-buffered saline and 1% Tween-20 (TBS-T) then incubated with respective antibodies. All antibodies were diluted in TBS-T with the following dilutions: 1:2,000 for rabbit polyclonal anti-TDG (GTX110473),

1:10,000 mouse monoclonal anti-FLAG (Sigma, clone M2), 1:2,000 mouse monoclonal anti-His6 (GE Life Sciences 27471001), 1:2,000 mouse monoclonal anti-Myc (Santa Cruz, clone 9E10), 1:5,000 mouse monoclonal anti-GFP (Living Colors, clone JL-8), 1:2,000 mouse monoclonal anti-SUMO1 (Life Technologies, clone 21C7), 1:5,000 rabbit polyclonal anti-SENP1 (courtesy of Mary Dasso, NIH), 1:500 rabbit antisera anti-SENP2 (Matunis lab, JH868), and 1:5,000 mouse monoclonal anti-tubulin (Sigma, clone DM1A). Most signals were detected with enhanced chemiluminescent substrates (Luminata Western HRP Substrates, Millipore). Signals that required sensitive intensity detection and analysis were detected and intensities measured and analyzed using Odyssey Imaging Systems (LI-COR Biosciences).

Immunofluorescence microscopy

HEK293T cells were cultured in 6-well plates containing glass coverslips and washed twice with 1X-PBS. Cells were fixed in 2 mL of 2% formaldehyde 1X-PBS for 30 minutes at 25°C. After washing once with 1X-PBS, cells were permeabilized in 2 mL of 0.2% TritonX-100 1X-PBS for 7 minutes at 25°C. After a final wash with 1X-PBS, coverslips were transferred to thick whatmann paper wetted with ddH_2O and maintained in the dark for antibody probing. All antibodies were diluted to 1:500 in TBS-T. The following antibodies were used: rabbit polyclonal anti-TDG (GTX110473), mouse monoclonal anti-SUMO1 (Life Technologies, clone 21C7), and mouse monoclonal anti-SUMO2 (Matunis lab, clone 8A2). DNA was stained with 4`,6-diamidino-2-phenylindole (DAPI). Images were acquired using Zeiss Observer Z1 fluorescence microscope with 100x objective and obtained using Zeiss AxioCam MRm camera and processed using AxioVision Software.

Genomic DNA purification and dot blot

HEK293T cells were grown to 95% confluency in 6-well plates and washed twice with 1X-PBS then scraped off with 200 µL 1X-PBS. Genomic DNA (gDNA) was then prepared from these samples using GeneJet Genomic DNA Purification Kit per kit instructions for mammalian cell culture (Thermo Scientific, cat. K0721). Purified gDNA was stored in -20°C. DNA concentrations of all samples were measured by NanoDrop 2000 (Thermo Scientific).

For dot blot sample preparation, 500 ng of purified gDNA was brought up to 87 µL with ddH_2O and denatured with 4.35 µL of 10 N NaOH for 10 minutes at 96°C. Immediately after denaturing, samples were neutralized with 10 µL of 10 M ammonium acetate giving a final gDNA concentration of 5 ng/µL stock solution. For dot blotting, two whatmann papers prewetted in 2X saline sodium citrate (SSC) buffer (300 mM NaCl, 30 mM trisodium citrate, pH 7) and a nitrocellulose membrane (0.45 µm, Bio-Rad, cat. 1620115) were assembled in the Hybri-dot manifold dot blot apparatus (Bethesda Research Laboratories, Life Technologies, 96-well plate, cat. 1050MM) with suction applied throughout the dot blotting procedure. All wells were pre-washed with 150 uL of 2X-SSC buffer. Dilutions of 0 ng (ddH_2O), 50 ng, 100 ng, or 200 ng of prepared gDNA samples were brought up to 50 µL with ddH_2O then added directly to the nitrocellulose membrane and subsequently washed with 150 µL of 2X-SSC buffer. Membrane was allowed to air-dry before being baked at 80°C for 1 hour. Membrane was then blocked with 5% dry milk in TBS-T and probed with 1:5,000 rabbit polyclonal anti-5-carboxylcytosine (Active Motif 61225). Signals were detected and intensities measured and analyzed using Odyssey Imaging Systems (LI-COR Biosciences). To ensure equal loading of gDNA on dot blot, membranes were washed with TBS-T after initial probing with anti-5caC antibody and incubated with methylene blue buffer (0.02% methylene blue, 300 mM sodium acetate pH 5.2) for 30 minutes at 25°C. Membrane was briefly destained with ddH_2O and imaged.

Results

Endogenous TDG is sumoylated to a high fraction *in vivo*

Previous groups have demonstrated that TDG is sumoylated in exogenously transfected cells and that both SUMO1 and SUMO2/3 paralogs can modify TDG by crystal-EM studies (Baba et al., 2005, 2006; Hardeland et al., 2002; Smet-Nocca et al., 2011). We sought to verify this observed sumoylation of TDG by our own cell biology techniques. Because most of the evidence for TDG sumoylation is based on exogenous or *in vitro* conditions, we also investigated the sumoylation status of TDG under normal, endogenous conditions.

We observed 55 kDa and 70 kDa bands when whole cell lysates of 293T cells were immunoblotted for endogenous TDG (Figure 4A, lane 1). Previous cloning and purification studies suggest the 55 kDa band to be unmodified TDG (Neddermann et al., 1996; Neddermann & Jiricny, 1993). To verify that the observed 70 kDa band is SUMO-modified TDG, we lysed cells with and without NEM. Consistent with previous sumoylation studies, when NEM is absent in lysis conditions the high molecular weight band of TDG is significantly reduced as compared to lysis conditions with NEM (Figure 4A, compare lane 2 to lane 1). This can be attributed to active SENP's in the conditions without NEM, as demonstrated by reduced SUMO1 high molecular weight smearing. Also of note, endogenous TDG in our immunoblots appears as a doublet in both the unmodified and modified forms. Two isoforms of TDG, isoform a and b, that differ in length by 24 amino acids in the N-terminus have been reported and may explain the doublet observed when probing for endogenous TDG (Takahashi, Hatakeyama, Saitoh, & Nakayama, 2005; Um et al., 1998). Next, we compared endogenous TDG sumoylation across three commonly used cell lines (HeLa, 293T, and U2OS) and found that TDG is modified to a high fraction, about 50%, in all cell lines (Figure 4, B and C). Because both

unmodified and modified forms of TDG were easier to analyze by Western blot in 293T cells, we performed the rest of our subsequent experiments in this cell line unless otherwise noted.

Additionally, we sought to determine co-localization of endogenous TDG with either SUMO1 or SUMO2 to verify that 293T cells observe similar TDG sumoylation patterns as reported previously (Moriyama et al., 2014). By immunofluorescence microscopy, we observe that TDG merges with both SUMO1 and SUMO2 within the nucleoplasm suggesting TDG co-localizes with both SUMO paralogs. (Figure 4D). We illustrate in the SUMO2 panel that endogenous TDG merges with SUMO2 puncta, which are reliable markers of PML nuclear bodies. This is in agreement with previous studies of transiently overexpressed TDG localization in other cell lines (Takahashi et al., 2005).

SENP1 selectively desumoylates TDG

Because endogenous TDG is sumoylated to such a high fraction, it is likely that dynamic TDG sumoylation is sensitive to disruptions in the deconjugating SUMO machinery. Because the SENP family of SUMO-specific proteases are the most abundant as well as best-studied isopeptidases (Kim & Baek, 2009), we sought to determine which SENP most affected TDG sumoylation. Additionally, we focused our studies on SENP1 and SENP2 as they have the most activity towards all SUMO paralogs and are differentially localized in the nucleus.

We transiently transfected siRNA oligos specific for SENP1 or SENP2 into 293T cells and determined the ratio of SUMO-modified TDG to total TDG by Western blot (Figure 5, A and B). In comparison to control cells, SENP1 knockdown doubled the fraction of sumoylated TDG. In contrast, SENP2 knockdown did not increase the fraction of sumoylated TDG. A double knockdown of both SENP1 and SENP2 recapitulated the SENP1 knockdown phenotype. While the total levels of TDG appear to change with

each condition, we only focused on the ratio of modified TDG to total TDG in order to address sumoylation. These results show that sumoylated TDG is sensitive to SENP1 and not SENP2 RNAi suggesting that TDG sumoylation is regulated by SENP1.

SENP1 catalytic domain is sufficient for efficient TDG desumoylation

In order to demonstrate that SENP1 regulates TDG sumoylation, we transiently transfected several constructs of GFP-SENP1 and GFP-SENP2 (Figure 5C) in cells and analyzed TDG sumoylation by Western blot. First, we addressed whether SENP1 or SENP2 overexpression in 293T cells shows a preference for deconjugating sumoylated TDG. Recapitulating the RNAi data, SENP1 overexpression decreased the modification of TDG while SENP2 did not (Figure 5D). Collectively, our knockdown and overexpression demonstrate that SENP1 specifically desumoylates modified TDG.

SENP1 is a multi-component protein containing a regulatory N-terminal domain from 1-417 residues and a catalytic domain from 417-644, with the catalytic cysteine at position 603 (Bailey & O'Hare, 2004). Because SENP1 is specific for desumoylating modified TDG, we wanted to determine the components of SENP1 required for its desumoylating activity towards TDG. In U2OS cells, we co-transfected FLAG-TDG with Myc-SUMO1 and different GFP-SENP constructs: full-length SENP1, catalytically inactive SENP1C603S, SENP1 N-terminal deletions (SENP1NΔ100, SENP1NΔ200, and SENP1NΔ300), SENP1 catalytic domain (SENP1cat), and two chimeras swapping the N-terminal or catalytic domains of SENP1 with the catalytic or N-terminal domains of SENP2 (SENP1N-2cat, SENP2N-1cat) (Figure 5C). We then analyzed TDG sumoylation by Western blot. TDG sumoylation is indeed dependent on SENP1 activity as catalytically dead SENP1 is unable to desumoylate modified TDG (Figure 5E). Additionally, the catalytic domain of SENP1 is sufficient for efficient desumoylation of sumoylated TDG. This is recapitulated by the observation that desumoylation of modified

TDG follows the catalytic domain of SENP1 of the two chimeras. Taken together, we demonstrate that SENP1 desumoylates both endogenous and overexpressed TDG. Moreover, only the catalytic domain of SENP1 is required for TDG desumoylation. While we cannot discount that the N-terminal regulatory domain of SENP1 contributes to targeting of the isopeptidase to modified TDG, the catalytic domain of SENP1 is sufficient and specific for TDG desumoylation based on our data.

TDG SIM mutant has decreased sumoylation compared to WT

The C-terminal SIM adjacent to the SUMO conjugation site in TDG has been demonstrated to interact in an intramolecular fashion to a conjugated SUMO protein (Figure 6B) (Smet-Nocca et al., 2011). This interaction is proposed to form a conformation change in the tertiary structure of TDG with the most notable implications in TDG binding to DNA and affecting repair activity as discussed earlier. However, there may be a consequence for this SIM interaction in relation to the stability of TDG sumoylation. We transiently co-transfected FLAG-TDGWT or FLAG-TDG SIM mutant (TDGE310Q) with Myc-SUMO1 in 293T cells and analyzed TDG modification by Western blot. Interestingly, we observed a substantial decrease in TDG sumoylation with the SIM mutant as compared to WT that cannot be explained by decreased SUMO1 expression levels (Figure 6A). This suggests that an aspect of dynamic sumoylation of TDG, either conjugation or deconjugation, is altered by the absence of the C-terminal SIM.

Monitoring 5caC accumulation allows for *in vivo* analysis of TDG BER activity

TDG BER activity has been well studied with *in vitro* systems, which have been the basis for determining substrates for TDG and their relative excision rates. Several studies measure TDG BER activity in cell culture but utilize exogenous reporter systems to

monitor glycosylase activity (Begley, Haas, Morales, Kool, & Cunningham, 2003; Bennett et al., 2006; Hardeland et al., 2002; Hu et al., 2010). Thus, measuring TDG BER activity *in vivo* for endogenous lesions has not yet been accomplished.

To measure TDG BER activity *in vivo*, we co-transfected TET1 catalytic domain (His6-TET1cd) plasmid with TDG wild type (FLAG-TDGWT) in 293T cells. We then monitored protein expression by Western blot (Figure 7, A and C) and 5caC accumulation by dot blot (Figure 7, B and D). We found that increasing amounts of TET1cd plasmid transfected alone results in a corresponding increase in 5caC accumulation, demonstrating that TET1cd transfection directly increases 5caC amount (Figure 7, A and B). Next, we show that the addition of TDGWT plasmids with TET1cd transfection decreases the TET1cd-dependent 5caC accumulation (Figure 7, C and D). The decrease observed in 5caC accumulation is dependent on the amount of TDGWT present in cells. Finally, we show that the TDGWT-dependent decrease in 5caC produced by TET1cd is absent in catalytically inactive TDG (TDGN140A) (Figure 7D, far right lane). These data show that TET1cd transfection induces accumulation of 5caC, an endogenous and specific substrate for TDG, and that we can analyze TDG activity by monitoring 5caC amounts by dot blot. We used this overexpression system and paired dot blot assay to measure TDG activity *in vivo*.

TDG sumoylation does not affect TDG BER activity

To investigate the effect of sumoylation regulating TDG BER activity *in vivo*, we used the TET1cd and TDG co-transfection system in 293T cells as described above. We co-transfected TET1cd with TDG sumoylation mutant (TDGK330R) or TDG SIM mutant (TDGE310Q) and monitored protein expression by Western blot and 5caC accumulation by dot blot. We compared the results from the sumoylation mutants to TET1cd co-transfection with TDGWT. We transfected a set amount of TET1cd across all constructs

and all conditions to obtain a similar amount of 5caC accumulation in order to compare TDG sumoylation mutants with TDGWT (Figure 8A). Additionally, we used increasing amounts of TDG constructs to discern potentially subtle differences in BER activity that may be attributable to sumoylation regulation. Importantly, we observed no deficit in TDGWT sumoylation when co-transfected with TET1cd as observed by Western blot and the continued appearance of the modified TDG band in TDGWT that does not appear in either sumoylation mutant (Figure 8A, comparing 80 kDa band to 55 kDa band). Thus, we treated the TDGWT co-transfection condition as a true positive control of endogenous TDG BER activity. 5caC dot blot results show no significant difference in 5caC accumulation between either TDGK330R or TDGE310Q compared to TDGWT (Figure 8B). Using only the 5caC intensities from the 200 ng dilution of the dot blot, we quantified 5caC and compared relative 5caC accumulations across TDG constructs. Sumoylation mutants have a similar decrease in 5caC levels with increasing TDG levels as compared to TDGWT (Figure 8C). Additionally, sumoylation mutants have similar, if not less, 5caC per amount TDG (Figure 8D). These data show that TDG constructs deficient in SUMO conjugation or SIM interaction are as robust as TDGWT in excising 5caC. This suggests that sumoylation does not play a role in regulating TDG BER activity in excising the endogenous substrate 5caC *in vivo*.

Discussion

Base excision repair is a key pathway in maintaining genome integrity and correcting DNA lesions from both exogenous and endogenous sources (Krokan & Bjoras, 2013; Sirbu & Cortez, 2013). Sumoylation is essential to normal cell function and has been shown to be critical in cell stress responses and DNA damage responses (Bettermann, Benesch, Weis, & Haybaeck, 2012; Bologna & Ferrari, 2013; Tempe, Piechaczyk, & Bossis, 2008; Yang & Chiang, 2013). Sumoylation has been proposed to regulate TDG activity by alleviating product inhibition at the abasic site, thereby allowing enzyme turnover and completion of the BER pathway. However, the evidence for the role of sumoylation in regulating TDG activity is based on *in vitro* using exogenous DNA legions and reporter assays to measure TDG activity. In order to address whether sumoylation affects TDG activity in a cell culture setting, we developed an *in vivo* assay utilizing an endogenous TDG-specific substrate as a marker of TDG activity. In addition, we characterized the desumoylation machinery of TDG in order to better understand the dynamic sumoylation process of the glycosylase.

We relied on cell culture transient overexpression studies of TET1cd with TDG constructs to directly assay TDG activity *in vivo* and to interrogate the role of sumoylation in regulating TDG activity. Recently, TDG has been implicated in an active DNA demethylation pathway whereby TET family proteins iteratively oxidize genomic 5mC bases to 5hmC and further oxidation products, the last of which is 5caC (Kohli & Zhang, 2013; Wyatt, 2013). Important to our studies, TDG-mediated BER pathway is the only known mechanism of removing 5caC in cells. Thus, 5caC is an endogenous substrate specific for TDG repair activity. Additionally, 5caC is easily monitored by dot blot and immunostaining using antibodies specific for the oxidized base. Finally, 5caC is a stable product as it is the fully oxidized form of 5mC and is not sensitive to stressful

assaying conditions such as alkaline or heat treatment. This is in contrast to the oxidation product just upstream of 5caC, 5fC, which is difficult to measure by dot blot due to its labile formyl-group and only confidently measured by mass spectrometry. We used these aspects of 5caC to our advantage in studying TDG regulation by determining 5caC was a suitable marker of TDG activity because of its stability and specificity. Importantly, while there are three genes within the TET family proteins, the catalytic domains are conserved and the genes are suggested to have some redundant activities. Thus, we used TET1cd as a means of achieving 5cac production. Using TET1cd overexpression, we increased the amount of endogenous 5caC present in 293T cells. Notably, we observed a dose-dependent increase in the amount of 5caC present when transfecting TET1cd alone and thus chose the TET1cd transfection amount that produced the middle range of 5caC accumulation to obtain a level sensitive to TDG activity. This level was then used in all subsequent experiments with TDG co-transfection. When co-transfecting TDGWT with TET1cd, our studies demonstrate that TDG does in fact repair 5caC as evidenced by the decrease in 5caC. Additionally, the more TDG present in cells results in a larger decrease in 5caC accumulation suggesting a dose-dependent response in TDG repairing 5caC. Collectively, our co-transfection system of TET1cd with TDG and subsequently assaying for 5caC accumulation by dot blot allows us to determine the robustness of TDG activity.

Using our TET1cd and TDG overexpression system and dot blot assay for 5caC, we compared conjugation and SIM mutants of TDG to WT to determine the role of sumoylation in regulating TDG activity. Our data show that both sumoylation mutants have activity towards 5caC as evidenced by the decrease in 5caC accumulation with increasing amount of TDG construct. Additionally, the sumoylation mutants appear to have at least comparable activity towards 5caC as TDGWT as demonstrated by similar decreases in 5caC with increasing TDG and similar amounts of 5caC per amount of

TDG. These data collectively suggest that sumoylation of TDG, either conjugation or C-terminal SIM interaction, does not regulate BER activity towards the endogenous TDG-specific substrate 5caC *in vivo.*

While our data appears to be in contrast to the established paradigm of sumoylation alleviating TDG product inhibition at the abasic, there are a few factors to consider that potentially hindered previous work and make our data unique. First, the basis of the current paradigm that sumoylation regulates TDG activity is based on *in vitro* assays measuring repair activity. The most used repair activity assay used by previous groups is the nicking assay (Hardeland et al., 2002). While optimized for enzyme activity, this assay importantly contains no SUMO machinery, either for conjugation or deconjugation. Thus, this assay is unable to determine the role of sumoylation in regulating TDG activity because it does not allow for dynamic SUMO signaling. To elaborate, if SUMO conjugation stimulates TDG dissociation from DNA, then it follows that active sumoylation by E1/E2/E3 cascade must occur only when TDG is bound to DNA. Additionally, in order for SUMO-TDG to participate in another round of BER excision, it must be deconjugated by an isopeptidase and returned to the unmodified state. Thus, a dynamic cycling between modified and unmodified TDG is required if sumoylation regulates TDG binding affinity to DNA. By not including either conjugation or deconjugation SUMO machinery, the nicking assay fails to accurately address whether or not sumoylation regulates TDG activity. Our assay overcomes these restraints by using a cell culture *in vivo* system to address the regulatory effect of sumoylation.

Our *in vivo* TDG BER activity assay was developed specifically to measure 5caC levels as an assay for TDG excision rate. Because we only measure 5caC, we are potentially ignoring differential TDG activity towards other known substrates, such as 5fC, G/U mismatches, and G/T mismatches. This is a plausible concern as it has been demonstrated that TDG activity towards G/U can be enhanced while leaving activity

towards G/T unaffected (Hardeland et al., 2002). Interestingly, SUMO-TDG has increased activity towards G/U compared to unmodified TDG by the nicking assay previously mentioned while this effect is not seen with G/T. However, due to technical constraints, such as the inability to measure 5fC by dot blot due to its labile chemistry, as well as biological constraints, such as redundant cellular repair mechanism to excise G/U and G/T, it is currently difficult to devise an *in vivo* TDG activity assay specific for other TDG substrates similar to ours.

An important consideration of addressing SUMO regulation of TDG activity by our *in vivo* activity assay is whether or not TDG is catalytically turned over. Specifically, it is unclear by our current data if the TDG sumoylation mutants exhibit turnover. We can assume that TDGWT appropriately turns over because it is the native protein. However, we cannot assume that TDGK330R or TDGE310Q turnover in the same manner. While we observe repair of 5caC with both sumoylation mutants, there are two potential mechanisms of repair. In one mechanism, the TDG sumoylation mutants behave as TDGWT and each individual TDG protein is able to repair multiple 5caC lesions via catalytic turnover. However, in a competing mechanism, the TDG sumoylation mutants do not behave as TDGWT yet appear to repair 5caC as avidly as WT due to the large number of TDG protein available in the cell, as a product of transient overexpression. In this instance, each individual TDG protein is not turned over at the abasic site but is able to substantially decrease 5caC levels because there is substantially more TDG protein available than 5caC lesions. So TDG is saturating and obfuscates whether catalytic turnover occurs. Fortunately, this concern is testable in a few different ways, two of which we will highlight here. First, if TDG is not turned over then downstream BER enzymes are not permitted to complete repair and the abasic site persists. If TDG sumoylation mutants create significantly more abasic sites in comparison to TDGWT, then this suggests that the sumoylation mutants are not being turned over. Treatment

with alkaline conditions and a simple COMET assay to determine double strand DNA breaks (DSBs) will readily determine the relative amount of abasic sites. Second, if TDG is not turned over at the abasic site then it is not dissociating from DNA and will remain DNA-bound. Catalytic turnover can then be addressed by analyzing the amount of chromatin-bound versus nucleoplasmic TDG between sumoylation mutants and WT.

Glycosylases require efficient catalytic turnover from the abasic site of DNA in order for the glycosylase to participate in another round of excision and more importantly to allow for the rest of the BER pathway factors, such as APE1, to complete repair. This is crucial to maintaining genomic integrity since the exposed abasic site has the potential to lead to DSBs. Additionally, poor glycosylase turnover may lead to inappropriate recruitment of downstream repair factors by either not recruiting factors at the correct time, thus leading to stalled repair and potential for DSBs, or by not recruiting the correct factors and leading to inappropriate nucleotide incorporation at the damaged site.

In addition to addressing the role of sumoylation in regulating TDG BER activity, we sought to determine the desumoylation mechanism of TDG to better understand its dynamic SUMO processes. Collectively, our data conclusively demonstrate that SENP1 is the main isopeptidase responsible for regulating TDG sumoylation and that the catalytic domain is sufficient for this effect. Interestingly, when we knocked down SENP1 by RNAi and analyzed endogenous TDG sumoylation, we observed a decrease in total protein as well an increase in the sumoylated fraction. Conversely, when we knocked down SENP2 by the same method, we observed an increase in total protein but no change in the sumoylated fraction. This result may be worth following up with further studies determining the role of SENP knockdown in TDG stability. While recent evidence suggests sumoylation does not play a role in TDG degradation (Slenn et al., 2014), the observed fluctuation in TDG amount could be due to binding partners or other hidden effects governed by SENPs. Another striking observation in our SENP studies was the

increased SUMO-modified fraction of TDG when overexpressing SENP1N-2cat chimera (Figure 5E, lane 8). In comparison to control overexpression with GFP only, SENP1N-2cat substantially increases the sumoylated fraction of TDG. This dominant negative effect is particularly striking when compared to the SENP2N-1cat chimera that shows complete deconjugation of SUMO-TDG. A possible explanation for this effect is that the N-terminus of SENP1 does indeed interact with SUMO-TDG but the absence of the SENP1 catalytic domain makes this chimera unable to desumoylate modified TDG. Instead, by interacting with SUMO-TDG by its N-terminus, this chimera in effect protects SUMO-TDG from deconjugation by endogenous SENP1, thus resulting in increased sumoylated fraction of TDG. Further studies determining the interacting regions of SENP1 and SUMO-TDG will help to resolve this unique observation.

TDG is a unique SUMO substrate because of its high sumoylated fraction. Most proteins that are modified by SUMO cannot be identified as such until extensive mass spectrometry and immunoprecipitation analyses are performed and sample preparations enriching for the modified protein are done. In contrast, endogenous TDG sumoylation is observed by simple Western blot analysis of whole cell lysate. The high fraction of TDG sumoylation is reminiscent of RanGAP, another protein with an abundance of sumoylation (Matunis, Coutavas, & Blobel, 1996). RanGAP is sumoylated to a high fraction because of its unique localization in the nuclear pore complex (NPC) allowing for protection from promiscuous deconjugation (Matunis, Wu, & Blobel, 1998; Zhu et al., 2009). While TDG is not localized to the NPC in the same manner as RanGAP, a similar mechanism may explain its high sumoylation fraction. We observed that the C-terminal SIM mutant of TDG is not as avidly sumoylated as WT. A possible explanation for this observation lies in the unique structure of TDG. Because the C-terminal SIM of TDG is adjacent to the sumoylation site, the conjugated SUMO protein on TDG interacts with its own SIM in an intramolecular manner (Figure 6B). This interaction has been previously

discussed as a mechanism for generating a conformation shift in TDG allowing for alleviation of product inhibition during BER activity. However, this intramolecular SUMO-SIM interaction may play explain the high SUMO-modified fraction of TDG by affecting either deconjugation of SUMO-TDG (Figure 9) or conjugation of unmodified TDG (Figure 10). If affecting deconjugation, the intramolecular SUMO-SIM interaction imparts a unique protein conformation that makes it distinct from unmodified TDG. This new conformation may only be recognized by specific isopeptidases for desumoylation and any non-specific isopeptidases will fail to desumoylate modified TDG. In contrast, without the intramolecular SUMO-SIM interaction due to loss of the SIM, all isopeptidases are able to desumoylate TDG and thus observed TDG sumoylation is much lower. Alternatively, the SIM may participate in sumoylating unmodified TDG by enhancing recruitment of Ubc9. By creating a more stable interaction with Ubc9 via SIM interaction, sumoylation of TDG is increased. However, if no SIM is present then Ubc9 is not recruited as avidly and results in inefficient sumoylation of TDG. This outlines a mechanism similar to RanGAP sumoylation yet distinct due to the proximity of the SIM and SUMO conjugation site. While most proteins are not sumoylated to such a high fraction as TDG, this could be a general mechanism regulating dynamic protein sumoylation with the appropriate SIM and SUMO conjugation site.

Conclusion

In conclusion, TDG is a unique SUMO substrate. We have shown that its sumoylation does not regulate its BER activity. However, this does not rule out a role for sumoylation in regulating TDG. Recent evidence suggests that TDG may play a role in regulating transcription and gene expression (Cortazar, Kunz, Saito, Steinacher, & Schar, 2007; Jia et al., 2014; Leger et al., 2014; Luhnsdorf, Epe, & Khobta, 2014; Muller, Bauer, Siegl, Rottach, & Leonhardt, 2014; Xu et al., 2014) that may be a consequence of its now established role in active DNA demethylation. Thus, sumoylation may regulate the role of TDG in transcriptional activation as opposed to its BER activity. Additionally, we begin to identify the SUMO machinery contributing to the sumoylation state of TDG by demonstrating that SENP1 specifically deconjugates SUMO-TDG. Finally, we propose a general mechanism of proteins that contain a SIM proximal to a SUMO conjugation site may regulate sumoylation. Further exploring the processes required to dynamically regulate SUMO substrates will help to illustrate how sumoylation controls cellular functions.

Figure Legends

Figure 1. Base excision repair pathway.

The base excision repair pathway is the genomic repair mechanism that repairs single bases damaged by oxidation, alkylation, or deamination. Glycosylases specifically recognize and repair these DNA lesions, initiating BER by excising the damaged base and creating an abasic site. APE1 cleaves the deoxyribose backbone at the abasic site allowing for short patch (right branch) or long patch (left branch) pathways of nucleotide incorporation and ligation mediated by BER specific polymerases and ligases.

Figure 2. SUMO conjugation and SUMO interaction of TDG are proposed to affect its BER activity.

(A) TDG is a 410 amino acid residue protein consisting of two activity domains, the regulatory domain (RD) and the glycosylase domain. The regulatory domain mediates TDG interaction with dsDNA as well as determines G/T versus G/U mismatch activity. The glycosylase domain is conserved across UDG/MUG family glycosylases and utilizes a water-activating nucleophilic attack mechanism to break the C-N glycosidic bond of the damaged DNA base. TDG contains three sumoylation domains: N-terminal SIM (D133), C-terminal SIM (E310), and C-terminal conjugation site (K330). (B) Model of TDG catalytic core domain (blue) conjugated to SUMO1 (green) superimposed on dsDNA (yellow) demonstrating the steric clash (purple) that occurs between TDG and DNA due to the protruded α-helix (red) that forms due to TDG sumoylation. This image is adapted from Baba, Shirakawa et al, Nature 2005.

Figure 3. Iterative oxidation of 5mC by TET methylcytosine dioxygenases produces TDG-specific substrates, 5fC and 5caC.

5mC of genomic DNA is oxidized in a stepwise fashion to 5hmC, 5fC, and 5caC by the TET methylcytosine dioxygenase family of proteins (TET1/2/3). Each oxidation step requires a coordinating ferrous iron as well as adenosine triphosphate (ATP), 2-oxoglutarate (2-OG), and oxygen (O_2) and produces succinate and carbon dioxide (CO_2). Importantly, 5fC and 5caC are only repaired by TDG. This pathway is important in active DNA demethylation and is used in this study to generate TDG-specific substrates.

Figure 4. TDG is sumoylated endogenously.

(A) 293T cells were lysed by TNX100 buffer with and without 5 mM NEM and endogenous TDG sumoylation was measured by Western blot using antibodies specific to TDG, SUMO1, and tubulin. (B) Whole cell lysates from HeLa, 293T, and U2OS cells were probed for endogenous unmodified (55 kDa band) and modified (70 kDa band) TDG by Western blot using specific polyclonal antibody to TDG as well as tubulin. Two lanes per cell type. (C) Percent sumoylated TDG was calculated by measuring intensity of modified band and dividing by intensity of modified band plus unmodified band and normalized to tubulin. Error bars are SD; n=2. (D) Co-localization of endogenous TDG with endogenous SUMO1 (upper panel) or SUMO2 (lower panel) was imaged by fluorescence microscopy in 293T cells. Cells were probed with antibodies specific to TDG (dsRed, red channel), SUMO (GFP, green channel), or DAPI (blue channel) with corresponding merged image. White triangles denote overlap of TDG with SUMO2, which is also considered promyelocytic leukemia (PML) bodies. White bar indicates 10 μm.

Figure 5. SENP1 selectively deconjugates sumoylated TDG dependent only on its catalytic domain.

(A) 293T cells were treated with either non-specific (lane 1), SENP1 (lane 2), SENP2 (lane 3), or SENP1 and SENP2 (lane 4) siRNA oligos and endogenous TDG sumoylation was monitored by Western blot of whole cell lysates with antibodies specific to TDG, SENP1, SENP2, or tubulin. (B) Percent sumoylated TDG was calculated by measuring intensity of modified band and dividing by intensity of modified band plus unmodified band and normalized to tubulin. (C) Schematic diagram of GFP-SENP constructs used in overexpression transfections: wild types, chimeras, SENP1 catalytic mutant, and SENP1 N-terminal deletions; CAT is catalytic domain. (D) 293T cells were transfected with GFP (lane 1), GFP-SENP1 (lane 2), or GFP-SENP2 (lane 3) and endogenous TDG sumoylation was monitored by Western blot of whole cell lysates with antibodies specific to TDG and GFP. (E) U2OS cells were co-transfected with FLAG-TDGWT and different GFP-SENP constructs and TDG sumoylation was monitored by Western blot of whole cell lysates with antibodies specific to FLAG and GFP; note that FLAG blot is over-expressed TDG and unmodified and modified bands of FLAG-TDG are correspondingly shifted up in comparison to endogenous TDG.

Figure 6. Absence of C-terminal SIM motif decreases TDG sumoylation.

(A) 293T cells were co-transfected with Myc-SUMO1 and either TDGWT (lane 1) or TDG SIM mutant (TDGE310Q, lane 2) and TDG sumoylation was monitored by Western blot of whole cell lysates with antibodies specific to FLAG and Myc. (B) Surface representation model based on X-ray diffraction of TDG catalytic domain (TDG-CAT, gray) conjugated to SUMO1 (gold) with SIM's identified in red (SBM1 and SBM2) and contact surfaces between SUMO1 and TDG indicated in blue with specific emphasis on SUMO1 protein interacting with the proximal SIM2 of TDG. This image is adapted from Smet-Nocca, Benecke et al, BMC Biochemistry 2011.

Figure 7. TET-induced 5caC is repaired in a dose-dependent manner by TDGWT. 293T cells were transfected with (A and B) increasing amount of His6-TET1cd or co-transfected with (C and D) constant amount of His6-TET1cd and increasing amount of FLAG-TDGWT. (A and C) Protein levels were monitored by Western blot of whole cell lysates with antibodies specific to FLAG, His6, and tubulin. (B and D) Purified genomic DNA from corresponding Western blot samples were blotted by dot blot and 5caC levels monitored by specific polyclonal antibody. (C and D) Additionally, TDG catalytic mutant (TDGN140A) was compared to WT to identify TDG activity-dependent decrease in 5caC.

Figure 8. TDG sumoylation mutants have similar activity towards 5caC as TDGWT. 293T cells were transfected with constant amount of His6-TET1cd and increasing amount of FLAG-TDGWT, FLAG-TDGK330R (conjugation mutant), or FLAG-TDGE310Q (SIM mutant). (A) Protein levels were monitored by Western blot of whole cell lysates with antibodies specific to FLAG, His6, and tubulin. (B) Purified genomic DNA from corresponding Western blot samples were blotted by dot blot and 5caC levels were monitored by specific polyclonal antibody. Methylene blue staining was performed on dot blot membrane to ensure equal loading of genomic DNA. Additionally, a transfection containing no TDG construct was performed as a positive control of 5caC accumulation (TET only). (C and D) 5caC accumulation was analyzed for the 200 ng gDNA dilution samples of the dot blot. (C) 5caC was plotted against increasing normalized TDG and (D) 5caC per normalized TDG was determined for TDG protein levels of relatively equal abundance.

Figure 9. Model for intramolecular SUMO-interaction affecting target desumoylation.

SUMO-target desumoylation may be affected by the presence of a SIM proximal to the SUMO conjugation site. When sumoylated, the SUMO protein interacts in an intramolecular fashion with the proximal SIM to form a new tertiary conformation of the target. This new conformation partially buries the conjugated SUMO protein and protects the sumoylated target from spurious desumoylation. Only specific isopeptidases that can recognize this new conformation can desumoylate this protein. When the SIM proximal to the SUMO conjugation site is absent, the SUMO-target protein is unable to form the protected conformation and thus the SUMO protein is available for desumoylation by non-specific isopeptidases, resulting in decreased sumoylated fraction of the target protein.

Figure 10. Model for intramolecular SUMO-interaction affecting target sumoylation.

Target sumoylation may be affected by the presence of a SIM proximal to the SUMO conjugation site. There are a few possible mechanisms for this affect. If Ubc9 is not itself sumoylated (left path of Active SIM), the SUMO-thioester may interact with the proximal SIM of the target protein facilitating targeting and stability of the E2 interaction. The proximal SIM then “passes off” the SUMO-thioester to the conjugation site for sumoylation. If Ubc9 is sumoylated itself (right path of Active SIM), the SUMO protein conjugated to Ubc9 may interact with the proximal SIM of the target protein and in turn facilitating targeting and stability of the E2 interaction. The SUMO-thioester is then primed for conjugation to the target protein. Additionally, sumoylated adaptor proteins (not shown), such as E3s, may be involved in targeting Ubc9 and mediated by the target proximal SIM. When the SIM proximal to the SUMO conjugation site is absent, no additional complex stability is gained because of the absence of a SUMO-interaction thus leading to inefficient sumoylation of the target protein.

Figures

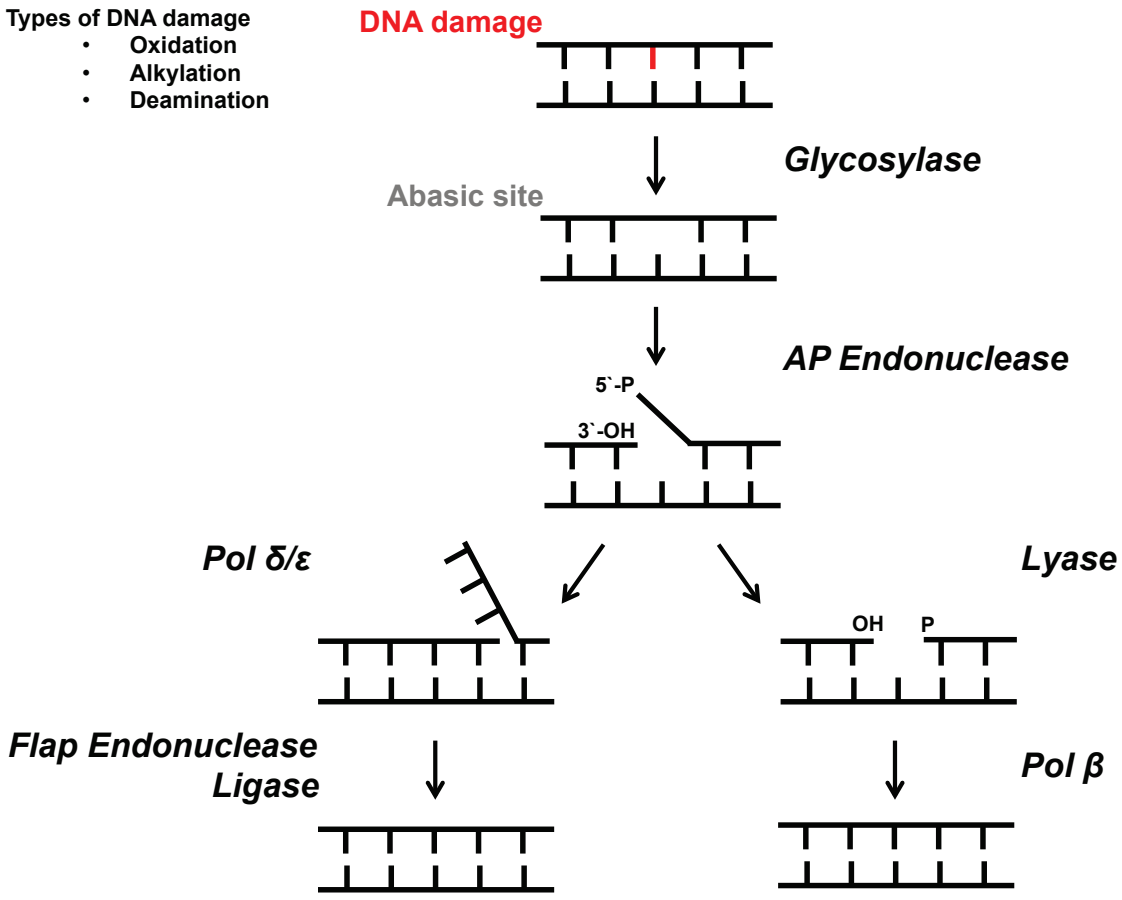

Figure 1. Base excision repair pathway.

2A.

2B.

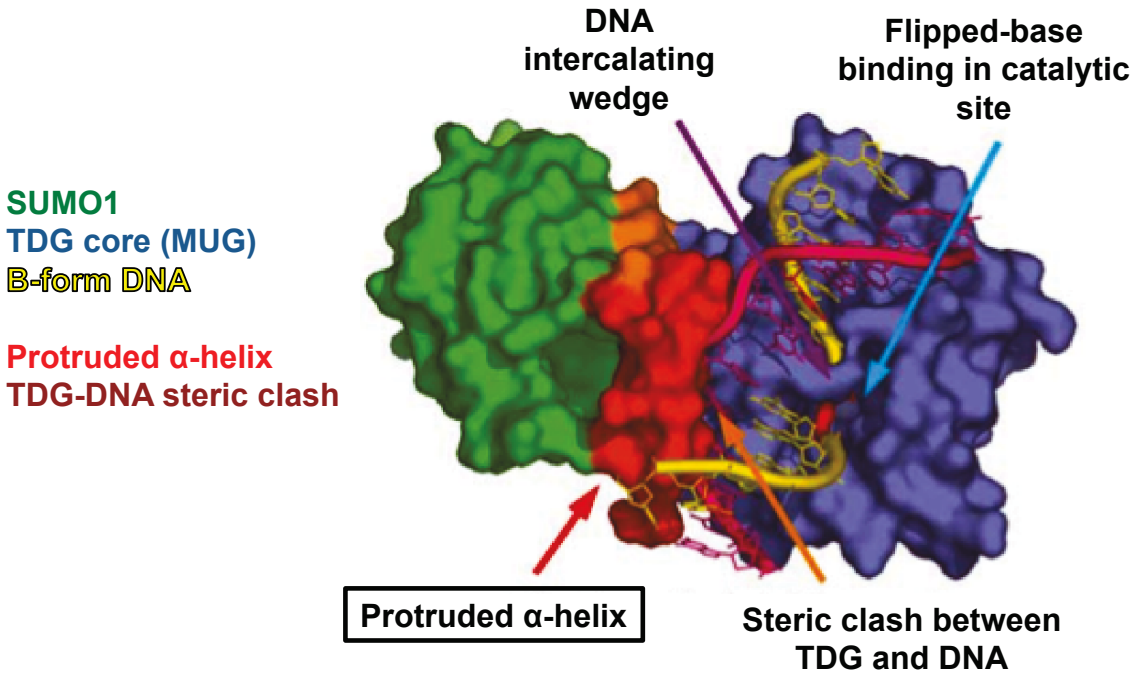

Figure 2. SUMO conjugation and SUMO interaction of TDG are proposed to affect its BER activity.

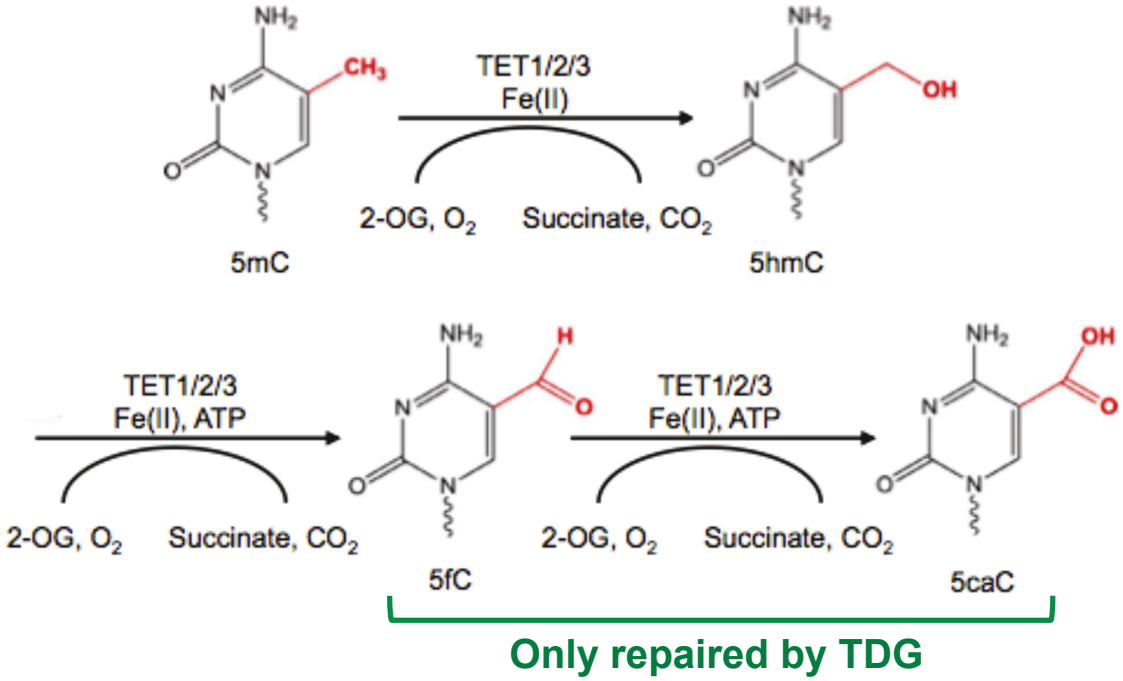

Figure 3. Iterative oxidation of 5mC by TET methylcytosine dioxygenases produces TDG-specific substrates, 5fC and 5caC.

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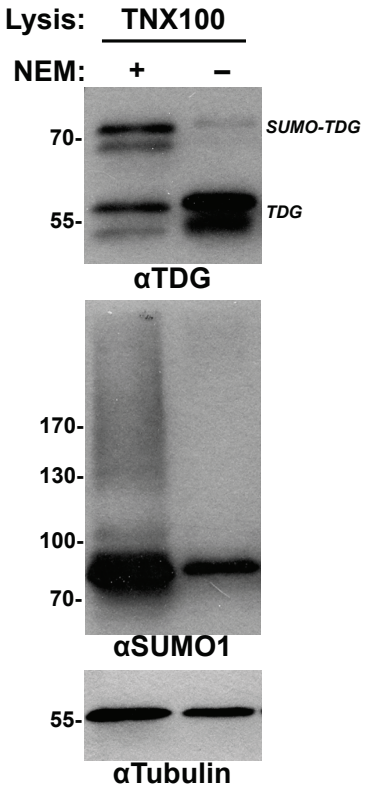
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SUMO-TDG
TDG
αTDG
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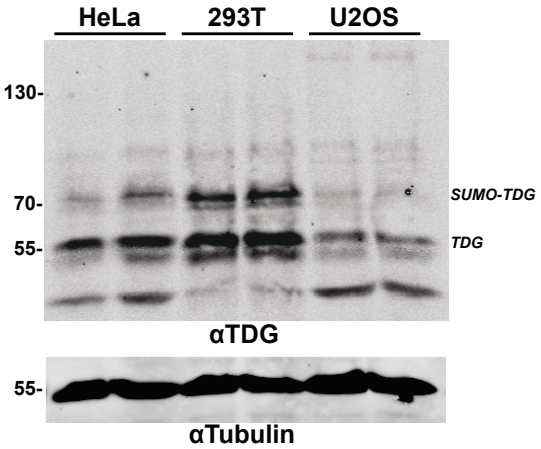
αTubulin

4B.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SUMO-TDG
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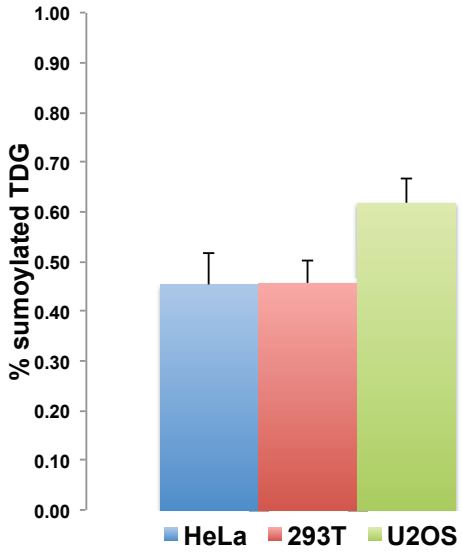
4C.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0.90
0.80
0.70
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0.50
0.40
0.30
0.20
0.10
0.00
HeLa
293T
U2OS

4D.

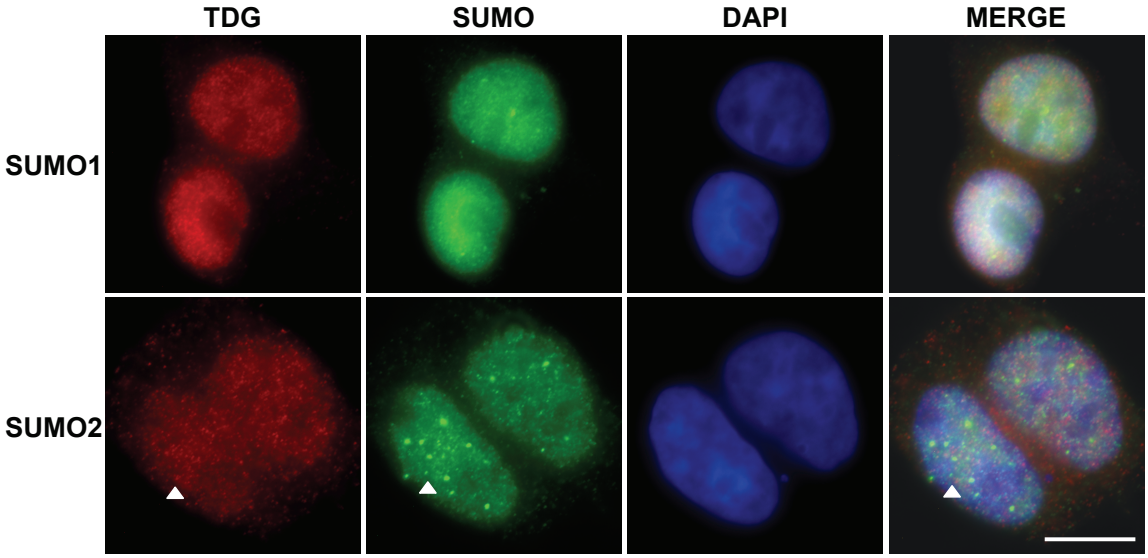
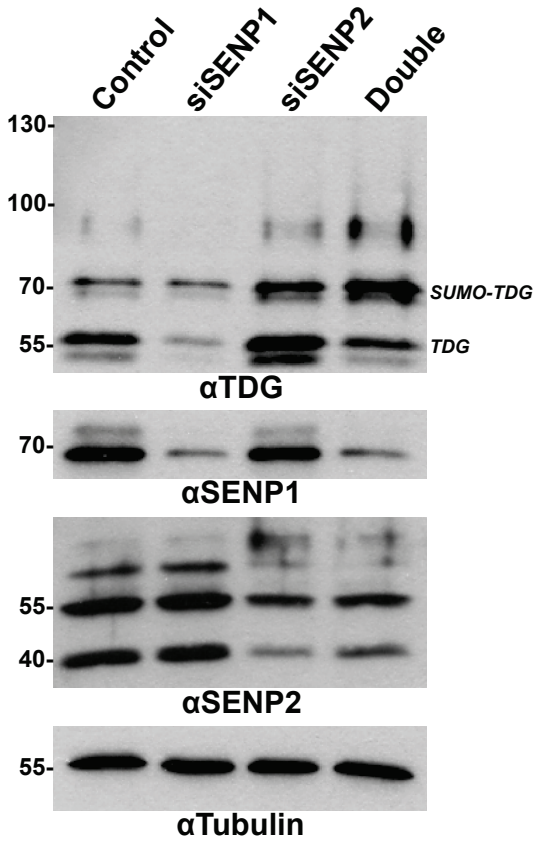


Figure 4. TDG is sumoylated endogenously.

5A.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siSENP2
Double
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100-
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SUMO-TDG
TDG
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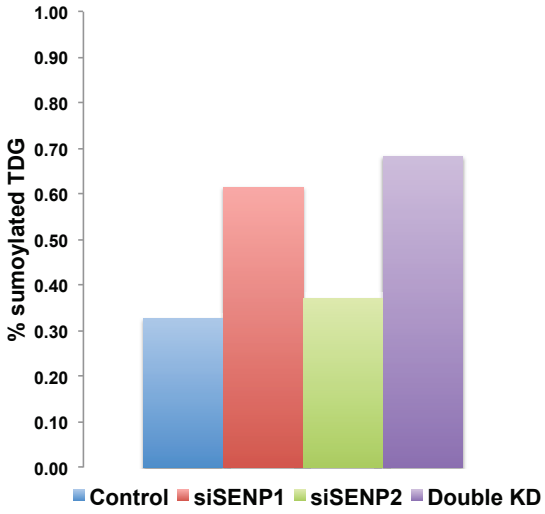
αSENP2
55-
αTubulin

5B.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0.90
0.80
0.70
0.60
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0.00
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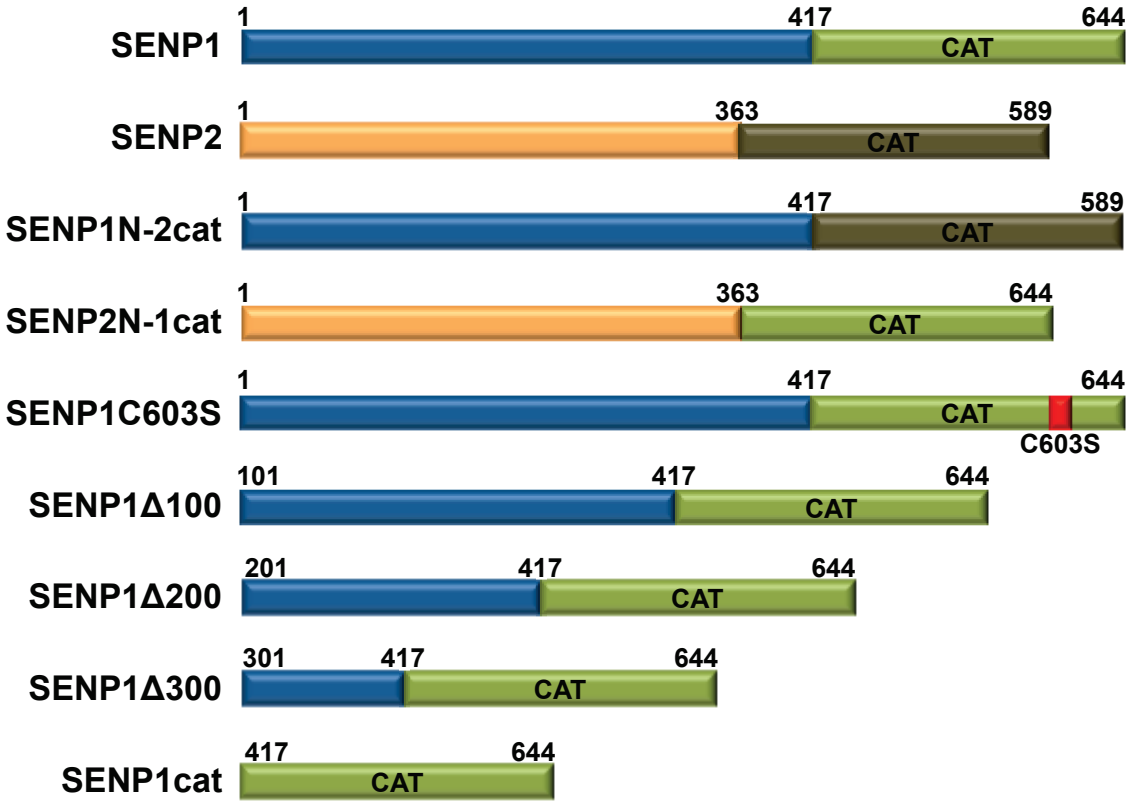
siSENP2
Double KD

5C.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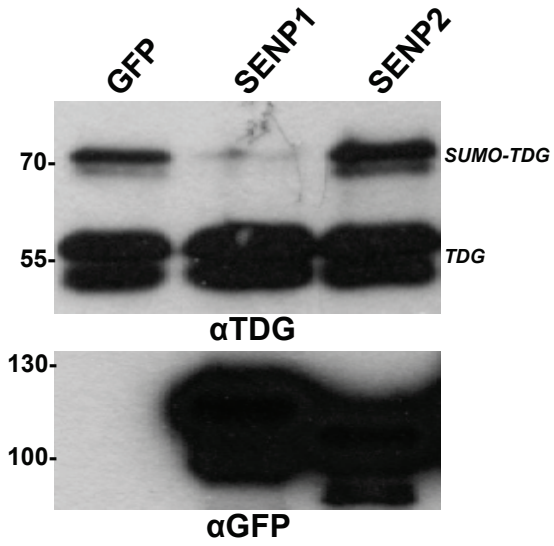
5D.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100-
αGFP

5E.

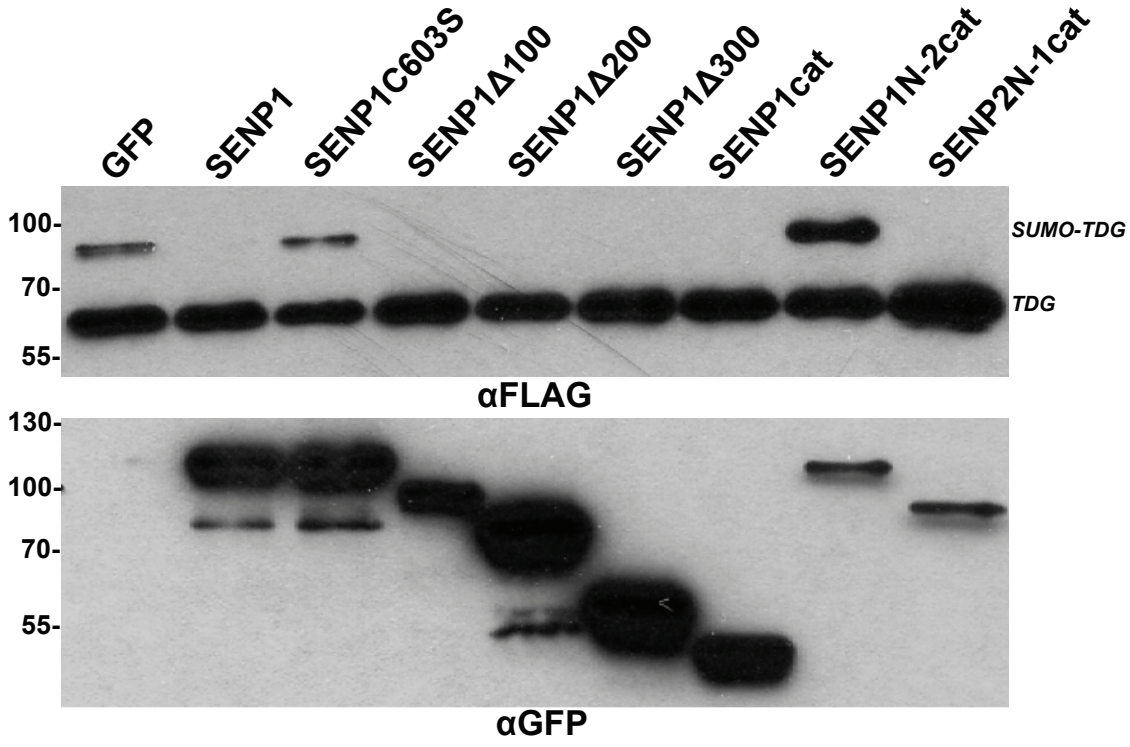
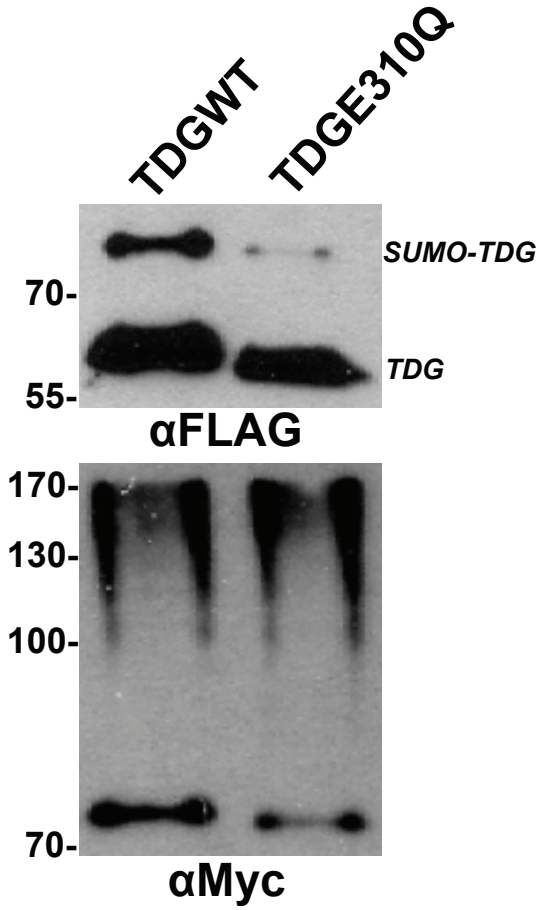


Figure 5. SENP1 selectively deconjugates sumoylated TDG dependent only on its catalytic domain.

6A.



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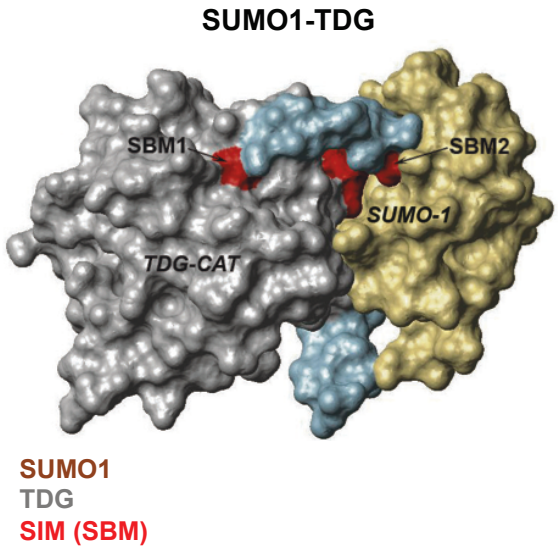


Figure 6. Absence of C-terminal SIM motif decreases TDG sumoylation.

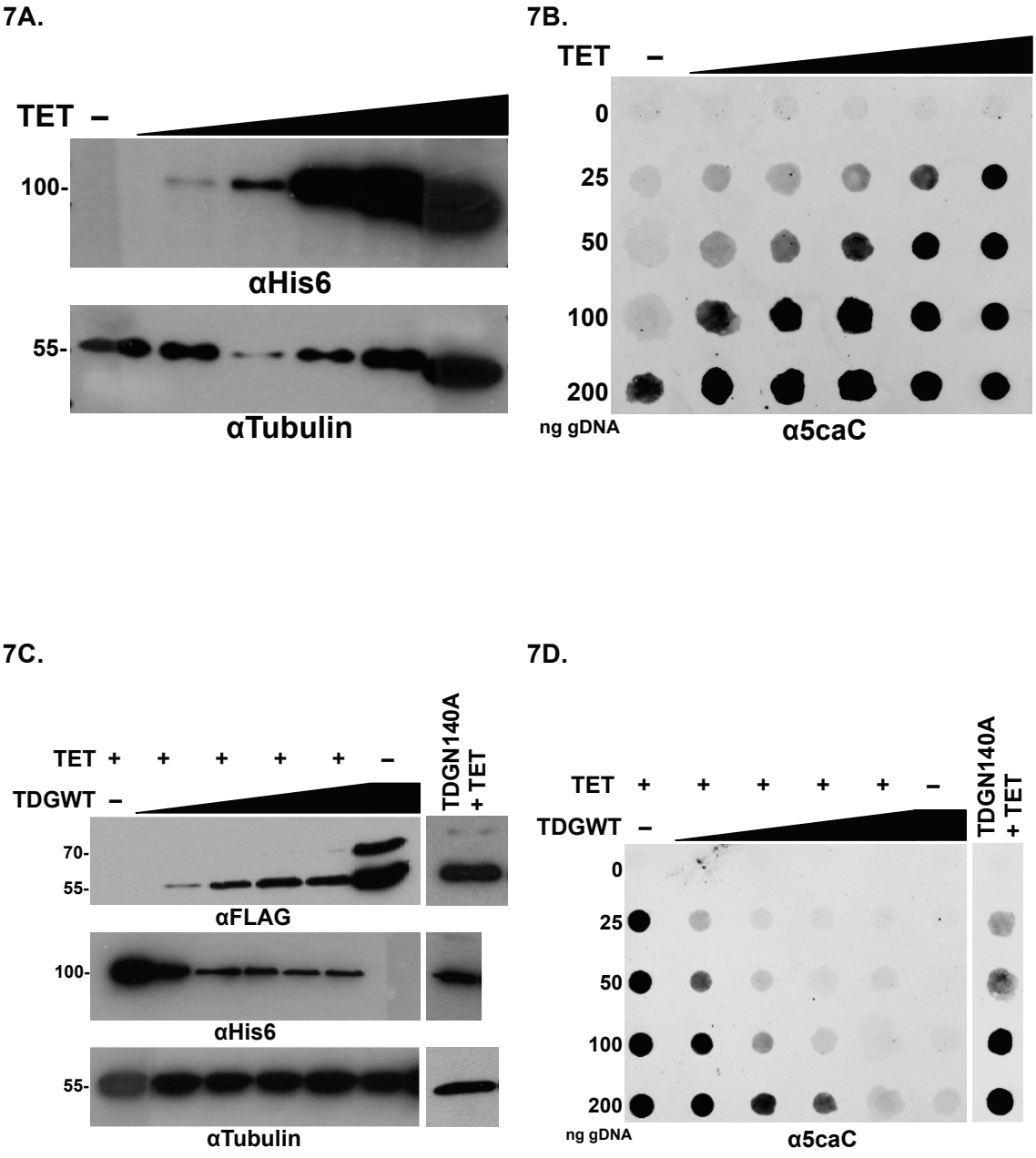
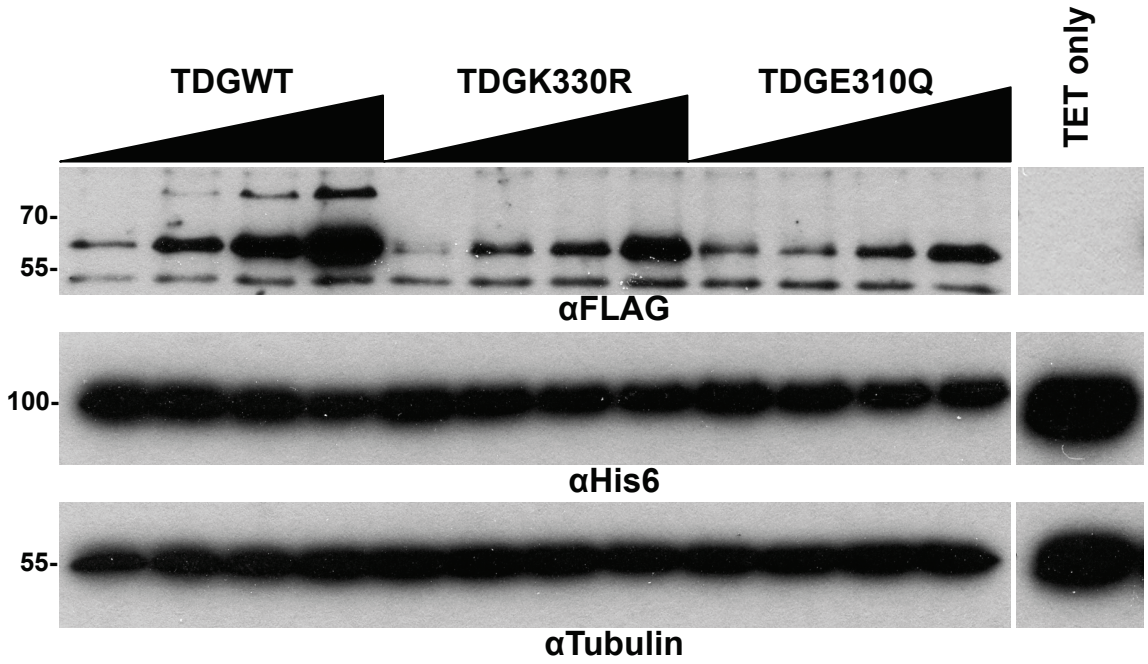


Figure 7. TET-induced 5caC is repaired in a dose-dependent manner by TDGWT.

8A.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TDGE310Q
TET only
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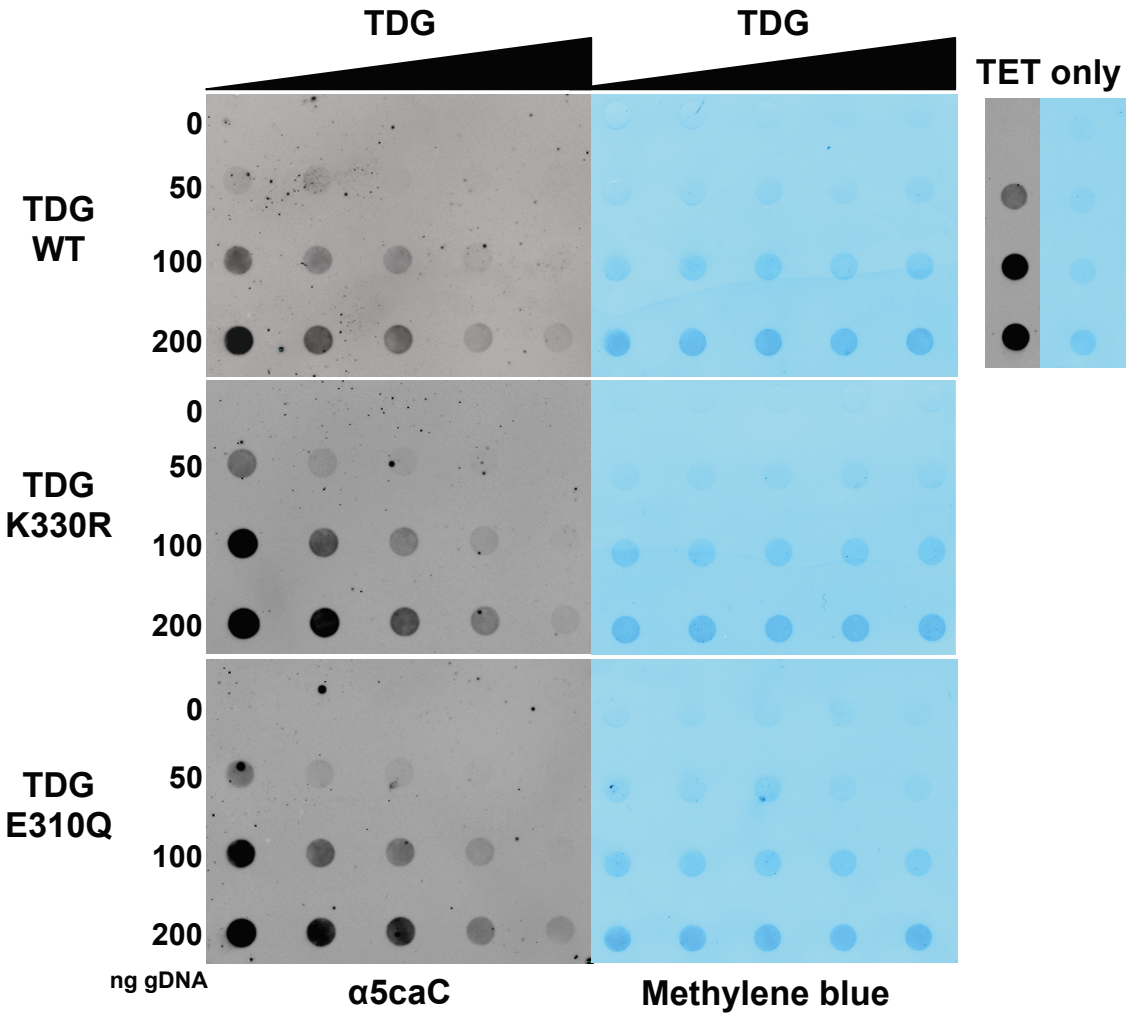
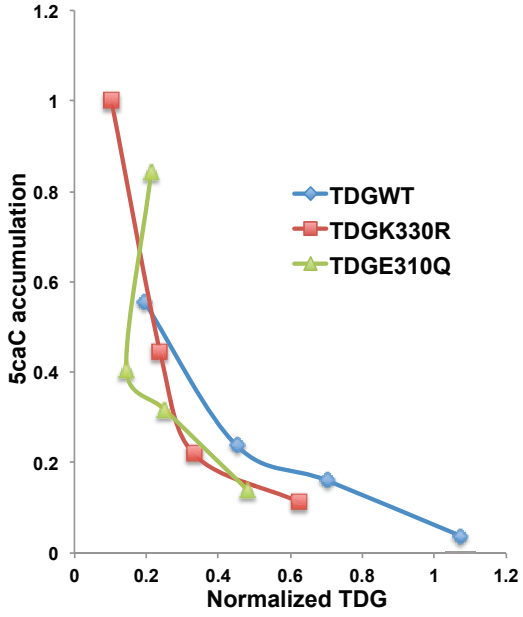
55-
αFLAG
100-
αHis6
55-
αTubulin

8B.


TDG
TDG
TET only
TDG WT
TDG K330R
TDG E310Q
0
50
100
200
ng gDNA
α5caC
Methylene blue

8C.



8D.

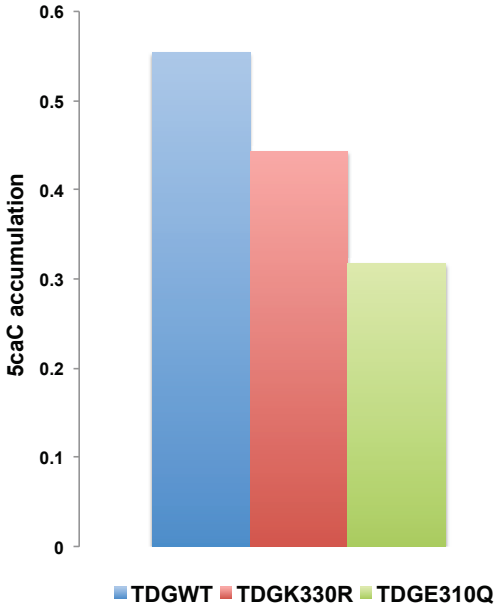


Figure 8. TDG sumoylation mutants have similar activity towards 5caC as TDGWT.

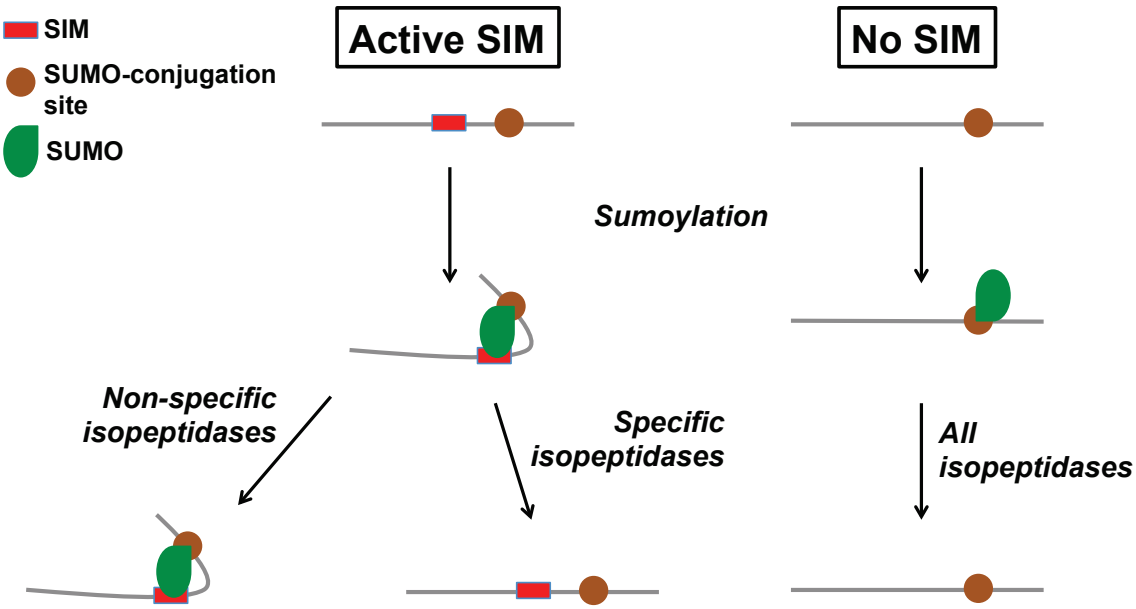


Figure 9. Model for intramolecular SUMO-interaction affecting target desumoylation.

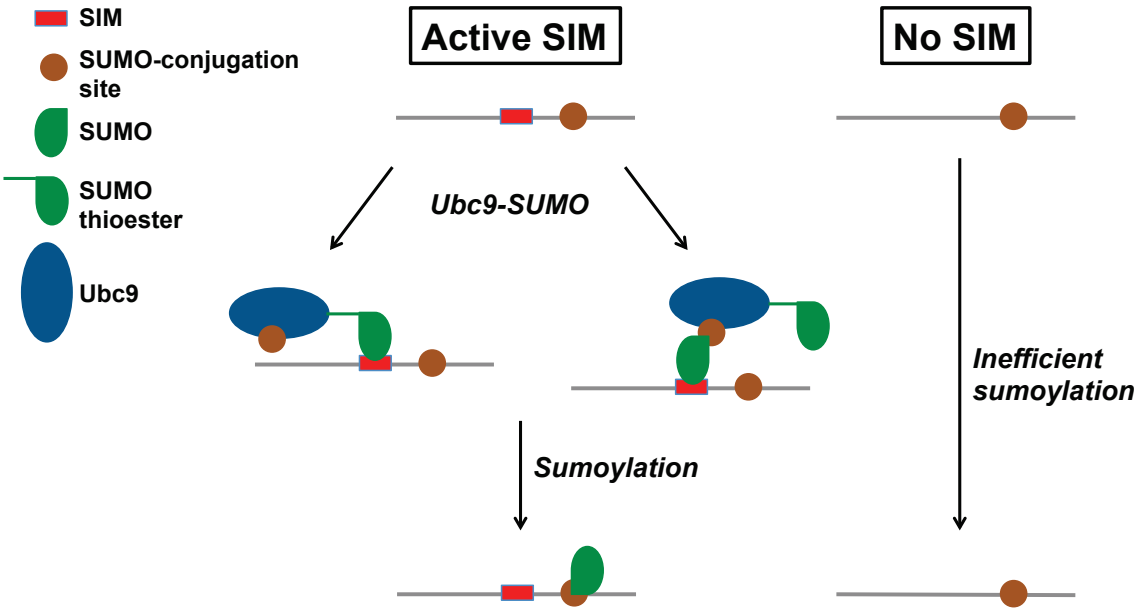


Figure 10. Model for intramolecular SUMO-interaction affecting target sumoylation.

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